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Q-PCR Based Bioburden Assessment of Drinking Water Throughout Treatment and Delivery to the International Space Station

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ABSTRACT

Previous studies indicated evidence of opportunistic pathogens samples obtained during missions to the International Space Station (ISS). This study utilized TaqMan quantitative PCR to determine specific gene abundance in potable and non-potable ISS waters. Probe and primer sets specific to the small subunit rRNA genes were used to elucidate overall bacterial rRNA gene numbers. while those specific for *Burkholderia cepacia* and *Stenotrophomonas maltophilia* were optimized and used to probe for the presence of these two opportunistic pathogens. This research builds upon previous microbial diversity studies of ISS water and demonstrates the utility of Q-PCR tool to examine water quality.

INTRODUCTION

Habitation of closed environments such as International Space Station (ISS) or those involved in long term space flights require that aspects of these environments be scrupulously monitored for agents that may affect astronaut health. To meet this requirement the Advanced Environmental Monitoring and Control (AEMC) element of NASA's Advanced Human Support Technology program developed a roadmap specifically addressing microbial contamination of manned spacecraft. Previous research has demonstrated that microbes exist in manned spacecraft and origins were human in nature (4, 25). However, these studies were culture dependent in nature and did not account for the total bioburden present. Since other studies have indicated that astronaut's latent viruses can reactivate after spaceflight (22, 24, 30) suggesting that their immune systems is under stress the need for a more complete picture of microbial contamination is essential. Therefore, it is critical to crew health to precisely monitor food, water and air for microbial contamination during habitation missions.

Recently culture independent DNA evidence has pointed to the presence of several opportunistic pathogens in samples taken from ISS drinking water or at various points along the treatment and delivery process (15). One of these opportunistic pathogen's, *Stenotrophomonas. Maltophilia*, rDNA was amplified from ISS water samples (14, 15). In addition, *Burkholderia. cepacia* like organisms have been isolated from post-flight shuttle tank water (unpublished results). These two organisms are of concern with respect to water supplies due to their ability to form biofilms (10, 13) and maintain resistance to a broad spectrum of antibiotics (20, 40). However, the presence of these bacterium in drinking water systems is not surprising because *B. cepacia* an *S. maltophilia* are ubiquitous in the environment (8, 20, 40) occupying a variety of niches from plant rhizospheres (20, 23) to rivers and well water (11, 12, 20). Unfortunately both have become common agents in nosocomial infections (28, 40) and have been implied in the contamination of hospital water supplies (10, 28). Perhaps more importantly they colonize and afflict immunocompromised patients (5, 12, 20, 28, 34) particularly those afflicted with cystic fibrosis (1, 2, 19, 37, 41). The insidious nature of these bacterium with respect to immunocompromised patients prompted further culture independent research to gain an understanding of their presence in ISS related waters.

The AEMC element has charged the scientific community with finding innovative technologies to identify and monitor microbes in the food, drinking water and air of closed habitats. This study examines the utility of TaqMan probes to distinguish DNA biomarkers of *B. cepacia* and *S. maltophilia* from the overall bacterial bioburden of ISS associated waters. TaqMan chemistries coupled with Q-PCR fluorescent detection is a technology that may in the future be coupled with microfluidic DNA extraction devices leading to a system that continuously monitors closed habitats on real time basis. Thus, flight managers will have another tool to protect crew member health.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. maltophilia* strain used in this study was obtained from the American type culture collection (ATCC# 13637). The *B. cepacia* strain was isolated from shuttle tank water following mission STS-113 (unpublished data). The strain was tentatively identified as *B. cepacia* after 16S rRNA gene sequencing and comparison to sequences in the GenBank database via a nucleotide blast search (http://www.ncbi.nlm.nih.gov\blast). Both strains were maintained on nutrient agar medium at 32°C. Strains of *S. africana*, *S. nitritireducens*, *S. rhizophila*, *S. acidaminipila*, *B. stabilis*, *B. pyrrocinic*, *B. anthina*, *B. vietnamiensis*, *B. ambifaria* were purchased from and grown as recommended by the ATCC.

DNA extraction from water samples. Water samples were collected during the STS-107, -113, -114 missions and analyzed at Kennedy Space Center (KSC) for cultivable microbes. Preflight samples consisted of Cocoa municipal water and deionized pre-biocide water that were collected at orbiter processing facility (OPF)-1. Post-biocide preflight samples were obtained from the shuttle's galley water chiller in the OPF-1 and at the launch pad. In-flight samples were obtained from the ISS drinking water dispenser (SVO-ZV) and from a port associated with the humidity condensate recovery system (SRVK-hot). Post flight samples were collected from shuttle supply tanks. All samples were maintained at 4°C, except in-flight samples which were maintained at ambient temperature until return to earth where they were stored at 4°C. DNA was extracted directly from each of 21 samples (20-100 mL) using a standard lysozyme boiling lysis followed by phenol/chloroform extraction protocol (26). DNA quantity was estimated spectrophotometrically (Nano Drop, Wilmington, DE) and generally found to be less than 2 ng per sample.

Design and testing of probes in-silico. Further phylogenetic analysis was performed using 16S rRNA gene sequences of *Burkholderia* and *Stenotrophomonas* species available in public databases. A maximum parsimony evolutionary tree (Fig. 1) encompassing 500 bootstrapped replicates was constructed using Phylogenetic Alignment Using Parisimony (PAUP) software (33). Primers universal to known eubacteria (32) were employed to enumerate the total number of 16S rRNA genes (Table 1).

Initially species specific probes and primers were designed based on previous work (17, 39). All available complete 16S rRNA sequences of organisms belonging to the genus *Burkholderia* and *Stenotrophomonas* were downloaded from GenBank http://www.ncbi.nlm.nih.gov/Genbank/index.html. The resulting sequences were aligned using clustalW algorithms within MacVector 7.2.2 (Accelrys Inc. San Diego, CA). Sections of homogeneity and heterogeneity were identified by eye and probes designed to anneal in variable regions. Probes were designed following parameters as recommended in the TaqMan Reagent Kit Protocol (Applied Biosystems, Foster City, CA): Tm of probes between 58-60 °C; Tm of primers less than probe and within 2-5 °C of each other; G+C content 30-80% avoiding stretches of G or C at the 3' end; amplicon length <400 bp. After a candidate probe was selected suitable primers were selected using the Primer Express (Applied Biosystems) program. The ability to produce amplicons was tested using the primer pair check within the MacVector program (Accelrys). In addition, probes were checked for specificity using Probe Match at the Ribosomal Database Project's web site (http://rdp.cme.msu.edu/probematch/search.jsp). All probes and primers used in this study are listed in Table 1.

Q-PCR conditions and optimization. Gene quantification was performed in triplicate using an MJ Research Chromo4 detection system coupled with a DNA Engine PTC-200 thermal cycler (Biorad, Hercules, CA). All primer and probe concentrations were optimized by trying all combinations of 4 different primer concentrations from 50 nM to 900 nM. The optimal concentration of primers was then used to test 5 different concentrations of probe ranging from 50 nM to 800nM. All samples were run in triplicate unless otherwise stated. The combination that yielded the lowest Ct and exponential amplification was chosen for further use. Initially annealing temperatures were set at according to published work but values returned by Primer Express were used for probes and primers designed for this study. The results of the optimization are summarized in Table 2.

Standard curves for all experiments were generated as follows: *B. cepacia* and *S. maltophilia* genomic DNA were subjected to PCR using 27F and 1492R primers. The conditions were as follows: 95°C for 1 min denaturation, 55°C annealing for 45 s, 72°C extension for 2 min repeated for 35 cycles. The amplicons were purified using Qiaquick columns (Qiagen, Valencia, CA) and cloned into the pCR-4 TOPO vector by TA cloning (Invitrogen, Carlsbad, CA), per manufacturers' instruction. Plasmids containing inserts were purified using Wizard minipreps system according to the manufacturers' instruction (Promega, Madison, WI) and quantified in ng/µL using spectrophotometry (A_{260}/A_{280}). The 16S rRNA gene copies/mL were calculated from the spectrophotometric results using the average molecular weight of a nucleotide and standard solutions serially diluted from 10^9 to 10^1 and used in each Q-PCR run. In addition genomic DNA was isolated from all the strains and used for primer specificity experiments.

Universal primers, 1369F 1492R, and the fluorescently labeled probe, TM1389F, were used to estimate the total copies of the eubacterial 16S rRNA gene in each sample. Each 50 µl reaction consisted of 25 µl of 2X Taqman Universal PCR Master Mix (Applied Biosystems Inc, Foster City, CA), 800 nM of each oligonucleotide primer, 500 nM of oligonucleotide probe, and 1 µL of template DNA. Reaction conditions were as follows: 95°C denaturation for 15 min., followed by 40 cycles of

denaturation at 95°C for 15 s and a combined annealing and extension at 60°C for 1.5 min. All other target amplifications were carried out using a 3 step PCR protocol. Each reaction contained Each 50 µl reaction consisted of 25 µl of 2X TaqMan Universal PCR Master Mix and 1 uL of template DNA. The optimal probe and primer concentrations are listed in Table 2. The reaction conditions were as follows: 95°C denaturation for 15 min., followed by 40 cycles of denaturation at 95°C for 20 s, annealing temperature as in Table 2 for 20 s and extension at 72°C for 30 s. After the completion of 40 cycles the reactions tubes were held at 4°C allowing for analysis of amplicons via gel electrophoresis if necessary.

RESULTS AND DISCUSSION

Probe and primer design. Initially a published probe and primer set, RWO1, DG74 and steno TM, was tested for specificity to distinguish *S. maltophilia* from the other *Stenotrophomonas* species (39). This probe and primer set yielded a signal for all the species of *Stenotrophomonas* purchased from the ATCC for this study. The studies of Wellinghausen et al. (2004) and others designed Q-PCR primers and probes to detect *S. maltophilia* from sputum and blood samples as well as to distinguish them from other clinical isolates (39, 40). Apparently genus specific probe and primers were enough to distinguish *S. maltophilia* in clinical samples as other species of *Stenotrophomonas* do not readily infect humans (5). However, with respect to the current work the necessity exists to distinguish this opportunistic pathogen from other organisms as they have been shown to co-contaminate water supplies (42, 43). Using an alignment of the 16S rRNA gene a variable region was selected and a probe designed using Primer Express. After optimization this probe and primer set was able to distinguish S. maltophilia from the other *Stenotrophomonas* strains used in this study (Fig 1.). S. maltophilia rRNA genes were 98% similar to *S. africana* but 96-97% similar to the other species Table 3. It is important to note that while the use of in-silico technologies greatly enhanced the speed at which the probes were designed, results from testing the probes ex-silico often yielded conflicting results. An interesting caviat is that the RDB Probe Check indicated that Sten TM would not anneal to any bacterium save one uncultured Xanthomonadaceae (Table 2) but in this study Sten TM yielded positive results for all the *Stenotrophmonas* species used.

Members of the *B. cepacia* complex maintain extremely similar 16S rRNA sequences (Fig. 1, Table 3) presenting a much greater challenge in finding a *B. cepacia* species specific rRNA probe. Over the past 20 years *B. cepacia* has emerged from a plant pathogen to a well recognized pathogen that colonizes the lungs of cystic fibrosis patients and certain strains maintain resistance to multiple antibiotics (18, 20, 36). Originally described by Burkholder in 1950 (3) as the causative agent in sour skin onion rot it was given the name *Pseudomonas cepacia* as cepia is Latin for onion. In 1997 taxonomic studies revealed that *B. cepacia* strains actually comprise a complex of 5 members (37). Research of the complex in the ensuing years revealed 4 more members of the complex bringing the total to 9 (6, 7, 35, 38). Members of the *B. cepacia* complex (Bcc) are phenotypically similar if not indistinguishable but genotypically distinct and thus endowed with genomovar designations from 1 to 9 (20). The species of *Burkholderia* are distinguished as follows (genomovar, *Burkholderia* species): I, *B. cepacia*; II, *B. multivorans*; III, *B. cepacia*; IV, *B. stabilis*; V, *B. vietnamiensis*; VI, *B. dolosa*; VII, *B. ambifaria*; VIII, *B. athina*; IX, *B. pyrrocinia* (20). It is now recognized that genomovar III is the most dominant isolate obtained from Bcc afflicted individuals (1, 9, 20) and based on DNA-DNA hybridization results there is a proposal to rename this genomovar *B. cenocepacia* (35).

A shuttle tank water isolate's (KSC Isolate; Fig 1.) 16S rRNA gene was completely bidirectional sequenced and a nucleotide-nucleotide blastn search (http://www.ncbi.nlm.nih.gov/BLAST/) indicated its identity as *B. cepacia*. However, the results shown in Figure 1 indicate the isolate was extremely similar to various members of Bcc. This was confirmed after the distances were placed in tabular format as % similarity (Table3) and revealed the KSC isolate was >98% similar to 7 members of Bcc. Even with this ambiguity a TaqMan probe was designed to single out *B. cepacia*. After optimization protocols probe Burk2P was able to detect the presence of the 16S rRNA gene from *B. vietnamiensis*, KSC Isolate (*B. cepacia*) (Table 2). *B. ambifaria* was also detected by this probe but a higher more stringent temperature of 58°C lowered its signal very close to background levels. An earlier probe design Burk1P was able to detect 16S rRNA genes from all the *Burkholderia* strains purchased for this study except *B. vietnamiensis*. Therefore, the probes Burk1P, -2P were used in unison to specify the presence of one *B. cepacia* like species (KSC isolate) 16S rRNA genes.

Analysis of ISS related water samples using Q-PCR.

Optimized probe and primer sets were employed to examine the 16S rRNA gene targets in samples from ISS related water. Eubacterial probe and primers produced trends very similar to previous cultivation and molecular analysis of the water samples (15). In general the highest number of targets was found in pre-biocide municipal water (Table 4). Deionization decreased eubacterial targets 2-4 fold and the addition of biocide decreased the targets another 1-2 fold. Samples taken after multiple additions of biocide and movement of the orbiting vehicle to the launch pad showed 0-1 fold decrease in targets from post-biocide pre-flight samples (Table 4). Samples taken from the drinking water ports aboard the ISS (SVO-ZV) maintained 10^4-10^5 targets while humidity condensate water (SRVK-hot) contained 10^5. Post flight analysis of waters remaining in the shuttles tanks yielded target numbers from 10^3-10^5 and B. cepacia (KSC isolate) was cultivated. Analysis of coolant loop waters indicated the presence of 10^4-10^5

targets. Species specific probes indicated *B. cepacia* like targets were much more prevalent throughout the system than *S. maltophilia* (Table 4). Previously S. maltophilia 16S rDNA was amplified and cloned from SRVK-hot and Q-PCR indicated 10^2 16S rRNA gene copies in this sample. Several samples contained 10^2 B. cepacia 16S rRNA gene copies however previous PCR and cloning failed to show the presence of these DNA markers. From these results several conclusions can be inferred. Iodine treatment is an effective biocide in these waters as shown by the decrease in cultivable bacteria and other molecular markers (14, 15) as well as 16S rDNA (this study).

This study is by no means all inclusive of the genera *Stenotrophomonas* and *Burkholderia*. In order to test the utility of TaqMan probes and Q-PCR technology to distinguishing various DNA targets found in drinking water.a limited number of readily available species were purchased and used. To ensure the validity of a usable probe, numerous strains from a variety of samples must be tested. One drawback in using Q-PCR as a true quantitative tool is the constitution of the standards. Suzuki et al. (32) address this issue and show that plasmid DNA over estimates the number of gene targets while linearized plasmids underestimate target numbers. In this study we employed rDNA cloned into plasmids as standards because of their ease of use and low cost. Since a major portion of this research was to find specific species in water samples then compare the numbers to overall bacterial targets the exact quantities were not critical.

CONCLUSION

Previously researchers have used 23S rRNA genes (40), enterobacterial repetitive intergenic consensus (ERIC) (5), restriction length polymorphisms (RFLP) (18, 21, 27, 29)and specific primers (17) to identify *Stenotrophomonas* and *Burkholderia* species from samples. However, these methods require the use of gel electrophoresis or subsequent DNA manipulations after isolation. Q-PCR was able to distinguish targets in a combined amplification and detection protocol. If the goal of the AEMC program is to move towards in situ detection of DNA or other biomarkers, then TaqMan and Q-PCR facilitate these needs. This study demonstrates the utility of the TaqMan system in the differentiation of gene targets in water samples. Further more TaqMan system is flexible enough to incorporate other DNA markers such at 23S or recA genes.

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Table 1. Probes and primers used in this study

Name	Primer or Probe	Sequence (5'-to- 3')	Position*	Optimal conc. (nM)	Annealing temp. used (°C)	Ref.
Universal eubacterial						
27F	primer	GAGTTTGATCMTGGCTCAG	11-27*	500	55	(16)
1492R	primer	AAGGAGGTGATCCANCCRCA	1492-1512*	500	55	(16)
Bact1369F	primer	CGGTGAATACGTTCYCGG	1369-1386*	800	60[2]	(31)
Prok1492R	primer	GGWTACCTTGTTACGACTT	1492-1510*	800	60[2]	(31)
RWO1	primer	TCGGAATCCTGCTGAGAGGC	1170-1189*	500	60	(39)
DG74	primer	AGGAGGTGATCCAACCGCA	1522-1540*	500	60	(39)
TM1389F	probe	CTTGTACACACCGGCCCGTC	1389-1408*	500	60[2]	(31)
B. cepacia						
PC-SSF	primer	TCGGAATCCTGCTGAGAGGC	994-1013*	800	60	(17)
BURK1F	primer	AACCTTACCTACCCTTGACA	928-947[a]	600	55	This study
BURK1R	primer	GGGATTCATTTCCTTAGTAAC	788-808[a]	600	55	This study
BURK2F	primer	GTGGATGATGTGGATTAATTCG	354-371[a]	600	55	This study
BURK2R	primer	GTGCGCCGGTTCTCTTT	893-914[a]	600	55	This study
BURK3F	primer	GCGTGTGTGAAGAAGGCC	354-371[a]	600	58	This study
BURK3R	primer	TGCTTATTCTTCCGGTACCG	435-454[a]	600	58	This study
BURK1P	probe	TCGGAATCCTGCTGAGAGGC	954-970[a]	300	55	This study
BURK2P	probe	TTGGCTCTAATACAGTCGGGGGA T	409-432[a]	300	58	This study
S.maltophilia						
STEN1F	primer	AACTGGAGGAAGGTGGGGAT	1145-1164[1]	600	60	This study
STEN2F	primer	TCCAGCCATACCGCGT	363-378[1]	600	57	This study
STEN2R	primer	CTTATTCTTTGGGTACCGTCATC	451-473[1]	600	57	This study
Steno TM	probe	CAGATCAGCATTGCGCGGTG	1313-1333*	500	60	(39)
STEN2P	probe	ATCCAGCTGGTTAATACCCGGTT	426-448[1]	300	57	This study

* Nucleotide numbering with respect to *E. coli*

[a] Nucleotide numbering with respect to *B. cepacia* 16S rRNA gene sequence (Genbank accession # AF335494)

[1] Nucleotide numbering with respect to *S. maltophilia* 16S rRNA gene sequence (Genbank accession # AB008509)

[2] A combination annealing and extension was used in a two step PCR reaction

Table 2. Specificity of probes used in this study

	Results of Probe Check from RDB				Experimental results 55°C		Optimized Temp	
Bacterium	**Sten TM**	***Sten2P***	**Burk1P**	**Burk2P**	**Sten2P**	**Burk2P**	**Sten2P**	**Burk2P**
B. athina	-	-	+	-	-	+	-	-
B. pyrrocinia	-	-	+	+	-	-	-	-
B. cepacia	-	-	+	+	-	+	-	+
B. ambifaria	-	-	-	-	-	-	-	+/-[a]
B. stabilis	-	-	-	-	-	-	-	-
B. thailandensis	-	-	+	-	-	ND	-	ND
B. pseudomallei	-	-	+	-	-	ND	-	ND
B. vietnamiensis	-	-	-	+	-	+	-	+
B. multivorans	-	-	-	+	-	ND	-	ND
B. cenocepacia	-	-	-	+	-	ND	-	ND
Herbaspirillum sp. G8A1	-	-	+	-	-	ND	-	ND
Herbaspirillum sp. NAH4	-	-	+	-	-	ND	-	ND
S. maltophilia	-	+	-	-	+	-	+	-
S. africana	+	-	-	-	-	-	-	-
S. nitritireducens	+	-	-	-	-	-	-	-
S. shizophila	+	-	-	-	-	-	-	-
S. acidaminiphila	+	-	-	-	-	-	-	-
Uncultured or unclassified bacteria	-	+	+	+	ND	ND	ND	ND
Uncultured Xanthomonadaceae	+	+	-	-	ND	ND	ND	ND

[a]*Amplification was not completely abolished but reduced to near background levels*

Table 3. Phylogenetic similarity based on boot strap distances

	KSC Isolate	S. acidaminiphila	S. africana	S. maltophilia	S. nitritireducens	S. rhizophia	B. ambifaria	B.anthina	B. brasilensis	B. caribensis	B. caledonica	B. caryophylli	B. cenocepacia	B. cepacia	B. fungorum	B. glumae	B. graminis	B. hospita	B. kururiensis	B. mallei	B. terricola	B. multivorans	B. phenazinium	B. phymatum	B.sacchari	B. sordidicola	B. stabilis	B. thailandensis	B. tropica	B. tuberum	B. ubonensis	B. unamae	B. vietnamiensis	B. pyrrocinia
KSC Isolate	100																																	
S. acidaminiphila	83.6	100																																
S. africana	83.2	96.0	100																															
S. maltophilia	84.0	96.9	98.5	100																														
S. nitritireducens	83.2	98.0	95.8	97.0	100																													
S. rhizophia	83.8	96.5	96.5	97.6	96.7	100																												
B. ambifaria	98.9	84.2	83.8	84.6	83.7	84.3	100																											
B.anthina	98.9	84.3	83.9	84.6	83.8	84.4	99.6	100																										
B. brasilensis	93.2	82.5	82.3	83.1	82.1	82.9	94.1	94.1	100																									
B. caribensis	94.1	83.6	83.1	83.8	82.9	83.8	94.7	95.0	95.2	100																								
B. caledonica	94.7	84.0	83.4	84.3	83.3	83.9	95.2	95.5	95.0	97.1	100																							
B. caryophylli	95.9	83.6	83.2	83.8	82.9	83.8	96.6	96.6	94.7	95.5	95.6	100																						
B. cenocepacia	98.5	84.3	84.0	84.7	83.9	84.5	99.3	99.5	94.0	94.7	95.2	96.3	100																					
B. cepacia	98.8	84.3	84.0	84.7	83.9	84.5	99.6	99.8	94.1	95.0	95.5	96.6	99.7	100																				
B. fungorum	94.5	83.5	83.1	84.0	82.9	83.4	95.1	95.3	94.6	97.0	98.6	95.5	95.0	95.3	100																			
B. glumae	97.7	84.3	83.8	84.6	83.8	84.3	98.5	98.4	94.2	94.2	95.2	96.3	98.8	98.6	95.1	100																		
B. graminis	94.3	83.8	83.1	84.0	82.9	83.5	94.7	94.8	94.6	96.9	98.5	95.2	94.5	94.8	97.4	94.7	100																	
B. hospita	94.5	84.0	83.4	84.2	83.4	84.2	95.2	95.4	95.4	98.7	97.1	95.8	95.2	95.4	97.1	94.9	96.8	100																
B. kururiensis	93.3	82.6	82.4	83.2	82.2	83.0	94.1	94.1	99.9	95.2	95.1	94.8	94.1	94.2	94.7	94.3	94.7	95.5	100															
B. mallei	97.1	84.1	83.7	84.6	83.5	84.1	97.8	97.9	94.2	94.4	95.4	97.0	97.9	97.9	95.1	97.7	95.2	95.1	94.3	100														
B. terricola	94.8	83.2	82.7	83.5	82.3	83.2	95.5	95.7	95.0	97.1	98.4	95.4	95.5	95.8	97.7	95.2	98.3	97.0	95.0	94.9	100													
B. multivorans	97.4	84.4	84.0	84.6	83.9	84.4	98.2	98.3	94.3	94.5	95.2	96.3	98.5	98.2	95.4	98.5	94.7	95.2	94.4	97.5	95.2	100												
B. phenazinium	95.2	83.7	83.3	84.1	82.9	83.5	95.8	96.0	94.5	96.9	98.0	96.0	95.7	96.0	98.4	95.9	97.0	96.9	94.6	95.7	97.6	96.5	100											
B. phymatum	94.1	83.5	83.0	83.6	82.8	83.5	94.8	95.0	96.4	98.2	97.1	95.7	94.7	95.0	96.9	94.7	96.3	97.9	96.5	94.7	96.6	94.7	96.5	100										
B.sacchari	95.0	83.8	83.6	84.3	83.1	84.1	95.6	95.8	96.2	95.5	95.5	94.7	95.5	95.8	95.5	95.5	95.5	96.0	96.3	94.9	96.0	95.9	95.5	95.4	100									
B. sordidicola	95.5	83.8	83.2	83.9	83.1	83.8	96.3	96.3	94.0	95.3	95.2	96.4	96.3	96.6	95.1	96.0	94.8	95.9	94.1	95.8	95.3	96.6	96.3	95.2	94.5	100								
B. stabilis	98.8	84.3	83.8	84.6	83.7	84.3	99.6	99.5	94.2	95.0	95.5	96.6	99.1	99.4	95.3	98.8	94.9	95.5	94.3	97.7	95.6	98.6	96.2	95.0	95.8	96.6	100							
B. thailandensis	97.2	84.2	83.7	84.6	83.6	84.2	98.1	98.0	94.6	95.0	95.5	97.0	97.9	98.2	95.4	97.8	95.2	95.3	94.7	99.0	95.3	97.4	95.8	95.1	95.2	95.8	98.0	100						
B. tropica	94.7	83.3	83.2	83.9	82.5	83.3	95.2	95.2	94.0	94.9	94.9	93.6	94.8	95.1	94.9	94.3	95.2	94.9	94.1	93.7	95.5	94.3	94.6	94.8	96.5	93.5	95.4	93.8	100					
B. tuberum	93.3	82.8	82.3	83.2	82.1	82.8	94.1	93.9	96.6	95.7	96.8	94.8	93.9	94.1	96.0	94.2	96.7	95.9	96.6	93.8	96.7	93.8	95.2	96.6	96.0	93.8	94.2	94.3	94.8	100				
B. ubonensis	98.2	84.1	83.9	84.5	83.7	84.3	99.1	99.0	94.0	94.4	95.0	96.3	99.4	99.1	94.9	98.6	94.5	94.9	94.1	97.9	95.5	98.5	95.7	94.7	95.3	96.0	99.0	97.9	94.8	93.8	100			
B. unamae	95.4	84.2	84.1	84.9	83.8	84.5	96.0	96.2	95.0	95.5	96.2	94.5	96.0	96.3	96.0	95.9	95.6	96.0	95.1	95.2	95.9	95.8	95.6	95.5	98.5	94.4	96.2	95.3	97.4	95.7	95.5	100		
B. vietnamiensis	98.7	84.0	83.6	84.3	83.5	84.1	99.3	99.3	93.8	94.5	95.0	96.2	99.0	99.3	94.9	98.1	94.5	94.9	93.9	97.4	95.2	98.2	95.6	94.4	95.5	96.1	99.3	97.7	95.1	93.8	98.7	95.9	100	
B. pyrrocinia	99.0	84.2	83.8	84.6	83.7	84.3	99.9	99.7	94.1	94.9	95.4	96.7	99.3	99.6	95.2	98.6	94.9	95.4	94.2	98.0	95.5	98.4	96.0	95.0	95.7	96.3	99.8	98.2	95.3	94.1	99.2	96.1	99.5	100

Sample Name	Source (mission; orbiting vehicle)	Total 16S rRNA gene copies per 100 mL	*B. cepacia* 16S rRNA gene copies per 100 mL (% total)		*S. maltophilia* 16S rRNA gene copies per 100 mL (%total)	
Cosoa municipal water						
OPF-1	Municipal water	2.8×10^8	0	(0.00)	0	(0.00)
OPF-2	Municipal water	3.8×10^7	0	(0.00)	0	(0.00)
Deionized pre-biocide water						
M1	STS-113; OV 105	2.0×10^6	0	(0.00)	0	(0.00)
C1	STS-114; OV 103	3.0×10^4	2.3×10^2	(0.77)	6.1×10^1	(0.21)
Post-biocide pre-flight water (at OPF)						
V2	STS-113; OV 105	1.2×10^4	2.6×10^3		0	(0.00)
V4	STS-113; OV 105	1.5×10^5	0	(0.00)	0	(0.00)
V6	STS-113; OV 105	7.5×10^4	4.7×10^2	(0.63)	0	(0.00)
Post-biocide pre-flight water (at launch pad)						
L-15; M2	STS-113; OV 105	1.8×10^3	6.0×10^1	(3.37)	0	(0.00)
L-3	STS-113; OV 105	2.8×10^4	0	(0.00)	0	(0.00)
L-3; MR2	STS-113; OV 105	3.2×10^4	3.3×10^2	(1.05)	0	(0.00)
L-15;M	STS-113; OV 105	1.0×10^4	2.2×10^2	(2.16)	0	(0.00)
L-3	STS-107; OV 102	1.4×10^3	3.0×10^2		0	(0.00)
ISS drinking water:						
SVO-ZV-I	STS-113; OV 105	1.3×10^5	0	(0.00)	0	(0.00)
SVO-ZV-1	Post	1.7×10^4	0	(0.00)	0	(0.00)
ISS humidity condensate water						
SRVK-hot-I	STS-113; OV 105	2.0×10^5	8.0×10^1	(0.04)	3.2×10^2	(0.16)
Post-flight shuttle tank water:						
PFC-1 Tank A	STS-112; OV 104	7.2×10^3	3.4×10^2	(4.65)	0	(0.00)
PFC-2 Tank B	STS-112; OV 104	1.49×10^5	0	(0.00)	0	(0.00)
PFM-1 Tank A	STS-113; OV 105	3.8×10^3	6.4×10^1	(1.75)	0	(0.00)
PFM-2 Tank B	STS-113; OV 105	3.2×10^3	0	(0.00)	0	(0.00)
Coolant Water (non-potable)						
Melfi	STS-114; OV 103	2.4×10^4	5.5×10^1	(0.23)	1.7×10^3	(7.19)
Express Rack #6	STS-114; OV 103	1.1×10^5	5.9×10^1	(0.05)	1.8×10^3	(1.68)

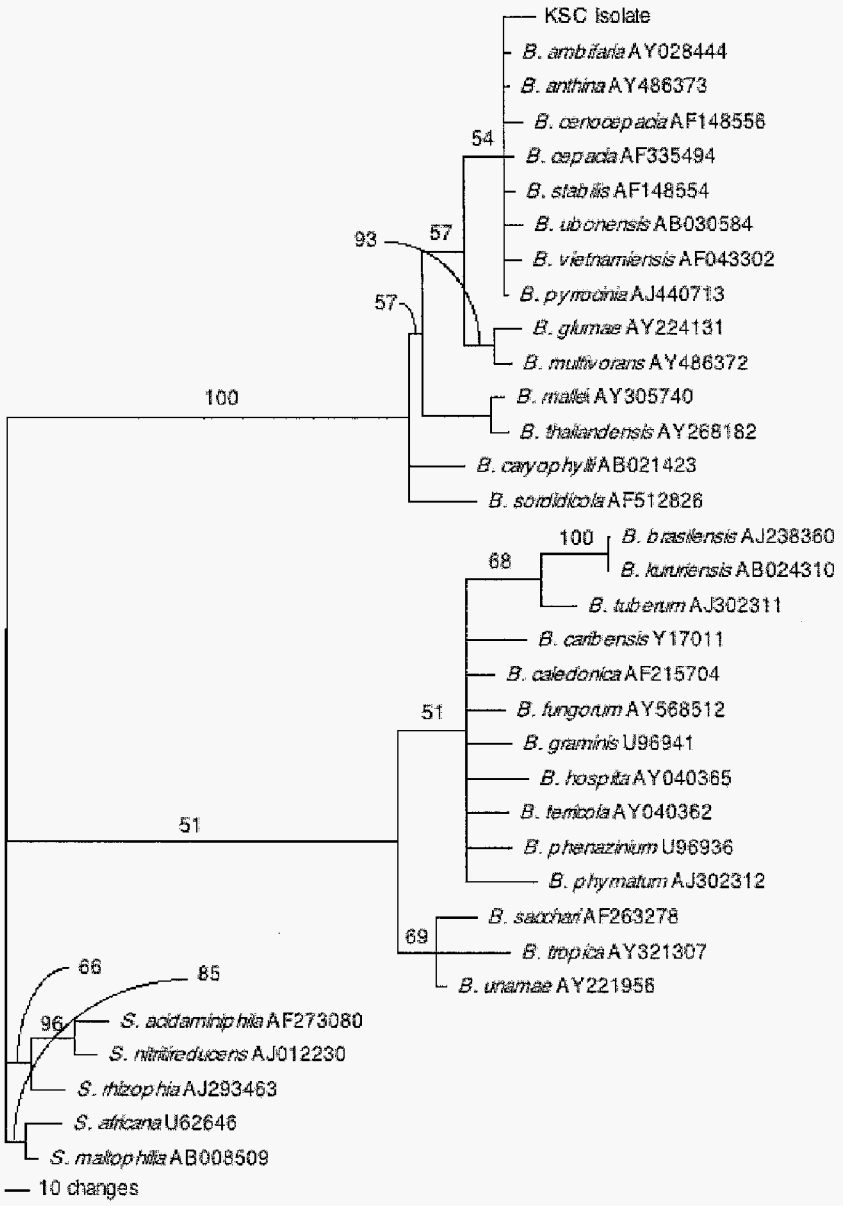

Figure 1. Phylogenetic tree of *Stenotrophomonas* and *Burkholderia* species 16S rRNA gene sequences. Sequences were downloaded from GenBank if the whole sequence was available (partial sequences were not used). The tree was constructed using maximum parsimony method, and genetic distances were computed by Kimura's model. The scale indicated te distance representatinv of 10 nucleotide changes. The numbers at the nodes indicated the percentages of occurrence in 500 bootstrapped trees.